Isolation of Clara Cells from the Mouse Lung

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A method is described for isolating Clara cells from the mouse lung that does not require the technique of elutriation. Mouse lungs totally perfused of blood are instilled with crystalline trypsin (0.25%) and incubated for the optimum time of 15 min. The lung tissue is chopped, mechanically agitated, and sequentially filtered to obtain a primary digest of 3 to 5 × 10^6 cells. Clara cells, identified routinely by histochemical localization of NADPH diaphorase, using the stain nitrotetrazolium blue (NBT), accounts for between 20 to 40% of the cells in the primary digest. Layering the cells of the primary digest on a discontinuous Percoll gradient followed by centrifugation gives rise to a major band of cells, 52% that are Clara cells (0.77 ± 0.28 × 10^6/mouse). A second method was devised to purify the Clara cells by simply centrifuging (32*g*, 6 min, 10 °C) the primary digest and discarding the supernatant that contained only a few NBT positive cells. When this process was repeated three times, the final pellet contained 68% Clara cells realizing 0.55 ±0.16 × 10^6 cells/mouse. The cells have typical Clara cell morphology as confirmed by electron microscopy and have a high level of P-450 enzymes (7-ethoxycoumarin deethylase and coumarin hydroxylase). Furthermore, the primary digests and the purified isolates contain less than 1% alveolar Type II cells, although such cells, identified by the histochemical localization of alkaline phosphatase, can be obtained by a second, more extensive digestion procedure. The simple procedure described for the isolation of mouse Clara cells could be further advanced if methods could be devised to prevent the loss of NADPH diaphorase activity during enzymatic digestion and cell centrifugation.

Introduction

The nonciliated bronchiolar or Clara cell has at least three roles in normal lung function: it contributes a secretion to the extracellular lining fluid, it is a progenitor cell for both itself and for ciliated cells, and it contains a variety of cytochrome P-450 monooxygenases that have an active role in the metabolism of xenobiotics (*1,2*). Of major toxicological interest is that the Clara cell acts as a progenitor cell for chemically induced lung adenomas (*1,3*) and, indeed, may be a specific target for a number of diverse chemicals (*4*). The primary role of the P-450 system is the detoxication of xenobiotics, but some chemicals may be converted to more toxic metabolites by monooxygenases. One example is the furan ipomeanol, a reactive metabolite that binds preferentially to Clara cells and is considered responsible for the potent pulmonary cytotoxicity observed (*5*). Isolated Clara cells have been used to study the metabolism of ipomeanol (*6*).

There are a number of advantages in working with isolated pure preparations of Clara cells. For example, they may be used to determine the potential specificity of chemical toxicity/targeting or to follow accumulation and binding of compounds. The simple manner in which the external milieu may be changed to block/stimulate the uptake of chemicals by isolated Clara cells provides a more adaptable system than that of working with the intact experimental animal. Pure isolates of Clara cells from untreated or chemically treated animals could be used to study normal or abnormal cell metabolism, the secretion of components, or the aspects of cell differentiation.

The potential realization of these important aims were considerably advanced by the pioneering studies of Devereux and Fouts (*7–9*) who isolated Clara cells from the rabbit. Their technique has also been used to obtain Clara cells from the rat (*10*). However, improvements to advance the original technique have not been forthcoming, perhaps because the methods are reasonably complicated and the purification procedure requires the use of an elutriator, which is not readily available in many laboratories. One further difficulty lies in the fact that the final yield of purified cells is very low (Table 1).

All of the isolation studies shown in Table 1 employ protease 1 as the digestive agent that is instilled intratracheally to release the primary population of cells. These cells are then purified by elutriation and gradient centrifugation.

There is scanty information on the loss of cells during the purification process with the exception of the early studies by Devereux and Fouts (*7,8*). They have shown that from a starting population of 290 × 10^6 cells/rabbit

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Table 1. Yields and purity of Clara cell preparations from the rabbit and rat.

Species	Percent of Clara cells in final fraction	Actual number of Clara cells in final fraction, $\times 10^{-6}$	Percent contamination with Type II cells	Reference
Rabbit	70	1.00	4.9	*(7)*
Rabbit	70	1.75–3.50	?	*(8)*
Rabbit	55	1.65	7.0	*(11)*
Rabbit	47	?	< 1.0	*(12)*
Rat	45	0.16–0.21	0	*(10)*

in the original digest, 14.5×10^6 were Clara cells (5% purity). Approximately 19×10^6 cells/rabbit were obtained from an elutriator fraction of which 5.7×10^6 were Clara cells (30% purity). Following a dextran/polyethylene glycol (PEG) gradient purification step, only 1×10^6 Clara cells/rabbit (70% purity) were obtained. Thus, following elutriation only 40% of the starting population of Clara cells remained, and after the gradient step this figure was reduced to 7%. An improvement in the cell yield ($1.75–3.50 \times 10^6$ Clara cells/rabbit) was reported when the PEG gradient was replaced with Percoll (*8*).

The aim of the present study was to investigate the isolation of Clara cells from the mouse lung. Since the investigators above showed that the yield of Clara cells was unlikely to be greater than 0.2×10^6/g lung wet wt, the choice of the mouse (lung wet wt 70–100 mg), would appear illogical. However, it has been reported that nonciliated cells are very numerous in the mouse bronchioles (*13*), a finding confirmed by Pack et al. (*14*) who reported that Clara cells in mouse lung form 50 to 60% of the cells of the airway epithelium. This indicated that the correct use of a primary digestion technique could release a relatively pure population of Clara cells. The use of mice was also considered important because of their previous use in a range of pulmonary toxicological studies particularly related to bronchiolar necrosis, with agents such as aromatic hydrocarbons (*15*), naphthalene (*16*), paraquat (*17*), or 3-methylindole (*18*).

A number of different approaches were used in the isolation of Clara cells from the mouse lung involving variations in the type of protease used, primary and secondary digestions of the tissue, and changes in the times of enzyme incubations. In most studies attempts were made to purify the cells using discontinuous or continuous Percoll gradients. Elutriation was not attempted because of the considerable cell losses experienced by earlier investigators. The best yields (approximately 0.5×10^6 Clara cells/mouse) and purity (Clara cells constitute 68% of the final population) were obtained using 0.25% crystalline trypsin and a simple centrifugation/washing procedure.

Materials and Methods

Animals and Materials

Male mice (CBA/H strain), usually aged between 8 and 13 weeks, were maintained on sawdust and given food and water *ad libitum*. Percoll was obtained from Pharmacia Ltd. (Middlesex, UK). Trypsin (Type I, T8003) and elastase (Type 2-A, 6883) were purchased from Sigma (Poole, Dorset, UK). DNase I (31135) was obtained from Fluka, Flurochem Ltd. (Derbyshire, UK), and dispase (241750) and collagenase/dispase (269638), from Boehringer Mannheim (Lewes, East Sussex, UK). All other chemicals used were of the highest grade available and were obtained from British Drug Houses, Sigma, or Boehringer.

Isolation Procedure

Mice were lightly anesthetized with halothane/air and given a lethal 1 mL IP injection of Nembutal/0.15 M NaCl (1:1 v/v) containing heparin (300 U/mL). Upon respiratory death the fur was washed with ethanol and then deflected from the skin, from the throat to abdomen on the ventral surface. The abdominal cavity was opened and the animal exsanguinated by severance of the major dorsal blood vessels. The trachea was exposed and a Luer cannula (Portex Ltd., Hythe, Kent; ref 200/300/030) was tied into place via a small incision at the top of the trachea. The diaphragm was carefully punctured to deflate the lungs, and the rib cage over the lungs and heart were removed. A second Luer cannula (ref 200/300/020) attached to a gravity feed of 0.15 M NaCl was inserted via a small incision in the heart so that it entered the pulmonary artery. The lung was then perfused free of blood with the saline that exited in a cut in the left atrium, while being artificially ventilated by air through a syringe attached to the tracheal cannula. With four to six ventilations the lungs should be perfectly white. The heart, thymus, and the rest of the rib cage were resected, and the lungs were removed from the cavity with the tracheal cannula still tied in place. The lungs were lavaged (4×0.6 mL of 0.15 M NaCl) via the cannula to remove free cells and pulmonary airway secretions and then lavaged once with the protease solution (usually 0.25% crystalline trypsin in solution *A* containing 133 mM NaCl; 5.2 mM KCl; 1.89 mM $CaCl_2$; 1.29 mM $MgSO_4$; 2.59 mM phosphate buffer, pH 7.4; 10.3 mM Hepes buffer, pH 7.4; and glucose at 1 mg/mL). The lungs were then refilled with fresh protease solution and suspended via a syringe attached to the cannula in 0.15 M NaCl maintained at 37 °C. Protease solution entered the lungs via the syringe by gravity feed and the level of the enzyme was maintained continually throughout the incubation period (30–40 mL required/mouse).

Following the incubation period (usually 15 min), the trachea and major bronchi were dissected free from the preparations and the parenchymal tissue was diced with scissors into 1- to 2-mm cubes. Trypsin activity was terminated by the addition of fetal bovine serum (1 mL/mouse), and the diced material (usually pooled from six animals) was suspended in solution *B* (solution *A* minus calcium and magnesium salts) containing 250 μg/mL DNase in a 50-mL plastic centrifuge tube. The suspension was repeatedly inverted by hand for 1 min to mechanically release cells from the tissue. The suspension was then filtered sequentially through gauze, 150 μm and 30 μm nylon mesh to obtain the primary cell digest, a sample of which was removed for preparation of cytospins and cell counting. In some studies the tissue remaining on the filters was removed and treated to a second protease digestion to obtain an additional population of cells. Cell suspensions from these primary or secondary digestions were then processed further by *a*) layering on a Percoll discontinuous gradient usually of density 1.040/1.089 (*19*) and centrifugation at 250*g* for 20 min at 4°C. A major band of cells at the 1.040/1.089 interface was removed and washed in solution *B* containing 50 μg/mL DNase; *b*) layering on a continuous Percoll gradient and processing as described for *a*; or *c*) the technique that was finally adopted in which the cells (2×10^6/10 mL tapered centrifuge tube) from the primary digest were centrifuged very lightly (32*g* for 6 min at 10°C) in 4 mL of solution *B* containing 250 μg/mL DNase. This step was repeated two additional times, and cell counts and cytospin preparations were made of the cells remaining in the supernatants 1–3 and of the cells recovered in the final pellet from the third centrifugation. The pellet of the final centrifugation contained a purified population of Clara cells that could be resuspended and used directly. If the cells were required for culture, gentamycin (50 μg/mL) and antiPPLO reagent (60 μg/mL) were included in solution *B* at each of the wash steps described above, and the final fraction of Clara cells was purified further by differential adherence in a culture medium of DCCM1 medium (Biological Industries Ltd, Glasgow, UK). Clara cells did not adhere to a plastic substratum over a period of 2 hr in this medium, whereas other cells such as macrophages/fibroblastlike cells readily attached.

Cell Identification

Cytospin (Shandon Instruments) preparations were stained specifically for Clara cells by the nitrotetrazolium blue (NBT) technique as described previously (*7*). The NBT stain is reduced in the presence of the enzyme NADPH diaphorase to an insoluble purple formazan. The NADPH diaphorase (also called a tetrazolium reductase) is thought to be located in the microsomal fraction of cells, and in early studies (*20*) it was considered to be a NADPH-cytochrome *c* reductase. A number of more recent studies (*4*) have confirmed that Clara cells contain NADPH-cytochrome *c* (P-450) reductase; other work (*21*) suggests that the reductase enzyme is sited between cytochrome P-450 enzyme(s) and that the whole complex interacts closely with the phospholipids of the endoplasmic reticulum. While the majority of lung cells contain some diaphorase enzyme, the activity of the enzyme is very high in Clara cells, presumably because of their high complement of P-450 monooxygenases. Thus, functional Clara cells may be specifically identified (by purple formazan formation) in cell preparations that have been pretreated with 10% formalin for 30 sec prior to NBT staining. Preparations were counterstained with 1% methylene green and mounted in glycerin. The number of functional Clara cells and the purity of the preparation (percent of the total population staining purple) was calculated by counting 1000 to 1500 cells from each cytospin. As the distribution of Clara cells was often uneven throughout the cytospin preparation, counts were taken at a number of points along the median diameter of the preparation.

In some experiments, a distinction was made between Clara cells that stained an intense purple (strongly NBT positive) with other Clara cells that did not stain as strongly. Presumably, any loss in staining intensity indicates a reduction in NADPH diaphorase activity and potentially a loss in functional P-450 activity. The location of Clara cells in the bronchiolar regions of the mouse lung was demonstrated by using 10 μm frozen sections that were subsequently fixed in 10% formalin for 1 min prior to staining with NBT. Cell samples ($4–8 \times 10^6$) were prepared for electron microscopy by 1% glutaraldehyde fixation, postfixing in 1% osmium tetroxide, incubating in uranyl acetate, and dehydrating prior to embedding in araldite.

Type II cells were also identified in cytospin preparations by means of a histochemical stain for alkaline phosphatase as previously described (*22*). Highly purified preparations of rat Type II cells which have high levels of alkaline phosphate activity (*19*) were used as controls.

Results and Discussion

Preliminary Lung Digestion/Clara Cell Separation Studies

In the mouse, Clara cells line the bronchiolar regions as shown by the specific localization of the NBT staining reaction (Fig. 1*a*) (*7*). Clara cells have a columnar shape, a nucleus located usually at the base of the cell, and on the luminal surface an apical cap region (*23*) is often present where fibrillar material is attached (Figs. 1*a* and *b*). Preliminary digestion studies with EDTA, elastase, and crystalline trypsin established that the latter enzyme (at a concentration of 0.25%) released most cells in the primary digest and was, therefore, employed in all subsequent isolations (Table 2). Between 40 to 60% of the primary digest cells could be recovered in a major band at the 1.040/1.089 interface after centrifugation on a discontinuous Percoll gradient (Table 2). When the cell preparation of the primary trypsin digest was stained with NBT/methylene green (Fig. 1*c*) a number of individual

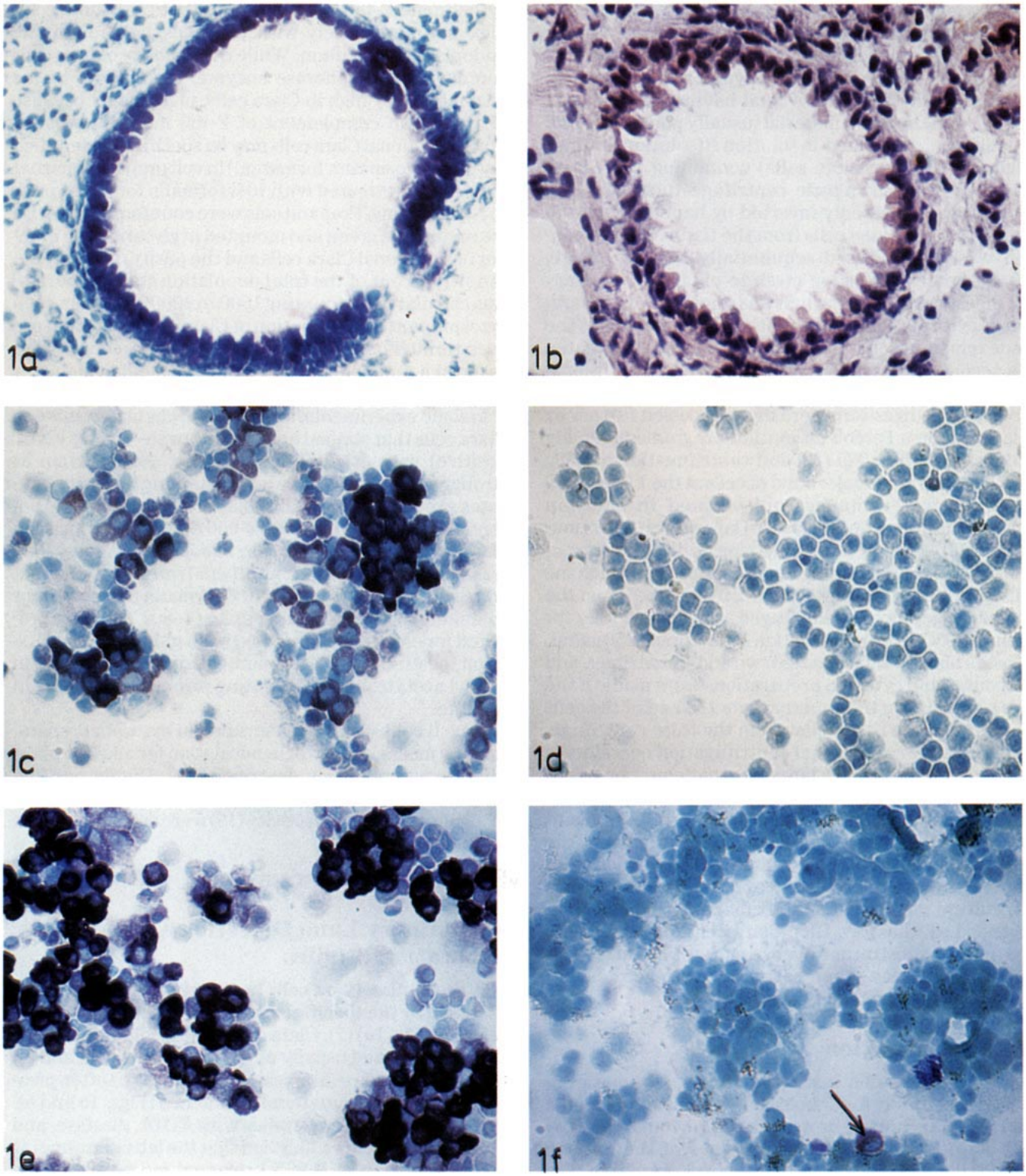

FIGURE 1. Identification and localization of mouse Clara cells. (*a*) NBT/methylene green staining of a frozen section of mouse bronchiole showing specific localization of the purple formazan deposit in Clara cells, ×250. (*b*) Hematoxylin and eosin stain of a wax section of the mouse bronchiole showing projecting apical regions of Clara cells, ×300. (*c*) NBT/methylene green stain of a cytospin preparation of cells of a primary digest derived by intratracheal instillation of 0.25% trypsin, ×250. (*e*) NBT/methylene green stain of the cells present in the major band of a discontinuous Percoll gradient, ×250. (*f*) Identical preparation of cells to (*e*) except that the preparation is stained for alkaline phosphatase (red deposit) and counterstained with methylene green. Note (arrow) the single alkaline phosphatase positive (Type II) cell present. A single mast cell that forms a deep blue color reaction with this stain is also present, ×250.

Table 2. The effect of different instilled agents on the total cells obtained from mouse lung following the primary digestion and separation on a Percoll gradient.[a]

Digestion agent	n	n_I	Cell yield × 10^{-6}/mouse: Primary digest	Major band on gradient	Percent recovery on gradient
EDTA, 2 mM	3	1	0.92	0.57	62
Elastase, 40 U/mL	3	1	1.63	0.95	58
Elastase, 20 U/mL	3	1	1.24	0.73	59
Trypsin, 0.25%	27	4	7.58 (± 7.22)	2.92 (± 1.11)	38

[a]Mice body weight 25 to 29 g; digestions for 30 min at 37 °C; n = total number of animals; n_I = number of separate isolations; the major band is found at the 1.040/1.089 interface; standard deviations (± SD) are given in parentheses where applicable.

and clumps of cells stained purple, indicating the presence of Clara cells. The most intensely stained cells were invariably those present in clumps. In contrast, free cells (mostly macrophages) derived by pulmonary lavage from the same mouse did not give a positive reaction with the NBT stain (Plate 1*d*). NBT staining of the cells in the major band of the Percoll gradient (Fig. 1*e*) suggested that this preparation had a higher proportion of Clara cells than the primary trypsin digest (Figs. 1*e* and 1*c*). Furthermore, the Percoll preparation was not contaminated with alkaline phosphatase positive cells (Fig. 1*f*), indicating that Type II cells were not present in the preparation.

Following the instillation of 0.25% trypsin, the time of incubation of the lung tissue was varied to determine if prolonged digestion would increase the yield of Clara cells (Table 3). A greater number of cells was found in the primary digest with increasing time of incubation, but proportionately fewer of these cells could be recovered in the major band of the Percoll gradient. Approximately 40 to 60% of the cells in the major band were NBT positive, but increasing the incubation time did not increase the Clara cell yield, although the preparation was more contaminated with alkaline phosphatase positive cells (Table 3). One further disadvantage of the prolonged (60 min) incubation with trypsin was the loss of strongly staining Clara cells. Intensely stained NBT cells are considered the most functionally competent and, as prolonged incubation with trypsin seemed to reduce diaphorase activity, the primary digestions with trypsin were limited to 15 to 20 min in subsequent isolations.

Distribution in and Purification of Clara Cells by Using Percoll Gradients

The preliminary studies indicated that a number of cells were not recovered in the major band (1.040/1.089 interface) of a discontinuous Percoll gradient. The distribution of total cells recovered and the proportions of these identified as Clara cells in such a discontinuous gradient are shown in Table 4. The majority of the cells recovered were found in the major band; 62% of these were Clara cells, and this band contained most of the cells which stained strongly with the NBT. Fewer Clara cells with reduced purity were detected in other parts of the gradient, and many of these cells did not stain intensely with the NBT. Therefore, while there was some loss of Clara cells by selection of the major band, the population of cells present in this fraction have the highest purity and an enhanced functional (diaphorase) activity.

A number of isolations were carried out using the methodology described previously to determine the value of purifying the primary digest by means of a discontinuous Percoll gradient (Table 5). The 55% recovery of Clara cells in the major band (0.77 × 10^6/mouse) from the number in the primary digest (1.41 × 10^6/mouse) was substantially higher than the equivalent recovery of the total cells (41%). Thus, the Percoll gradient step purified the cell population from 39% (± 3) to 52% (±7), but at the same time there was a loss of 45% of the starting population of NBT positive cells. Many of the cells that were not recovered would seem to be

Table 3. The effect of different incubation times (15–60 min) with instilled trypsin (0.25%) on the total yields of mouse lung cells and the number of Clara cells present in the major band of the Percoll gradient.[a]

Digest time, min	n	n_I	Cell yield × 10^{-6}/mouse: Total cells, PD	Total cells, MB	Clara cells, MB	Strong positive Clara cells, MB	Type II cells, MB
15[b]	44	6	2.89 (± 1.62)	1.23 (± 1.49)	0.63 (± 0.38)	0.36) (± 0.18)	0.111 (± 0.006)
20[c]	26	4	4.46 (± 1.15)	1.49 (± 0.38)	0.86 (± 0.24)	0.51 (± 0.14)	0.013 (± 0.011)
30[b]	9	1	3.88	1.47	0.63	0.40	0.014
60[b]	4	1	8.22	2.00	0.76	0.15	0.060

[a]PD = primary digest; MB = major band at interface of 1.040/1.089 Percoll discontinuous gradient.
[b]Age of mice 9 to 13 weeks.
[c]Age of mice, 28 weeks; remainder of key as for Table 2.

Table 4. Distribution of Clara cells in a discontinuous Percoll gradient.[a]

Gradient fraction	Cell yield × 10^{-6}/mouse				
	Total cells	Clara cells	Percent Clara cells	Strong positive Clara	Percent strong positive Clara
Band 1	0.70	0.16	21	0.07	11
	(0.61–0.80)	(0.13–0.18)	(21–22)	(0.07–0.08)	(10–12)
Major band	1.30	0.77	62	0.52	41
	(1.09–1.40)	(0.66–0.88)	(61–63)	(0.37–0.67)	(34–48)
Pellet[b]	0.15	0.22	13	0.01	6

[a]Mice body weight 30 g; lungs instilled with trypsin (0.25%) for 20 min incubation; band 1 is found at the suspension medium /1.040 interface, the major band is at the 1.040/1.089 interface and the pellet.

[b](Data from a single isolation) below the 1.089 fraction; primary digest contains 3.58 × 10^6 cells/mouse and the total recovery of cells on the gradient is 60%; results (with range values in parentheses) are from two experiments (n = 18 and n = 10, respectively).

Table 5. Purification of Clara cells from the primary digest using different discontinuous Percoll gradients.[a]

Sample/gradient	Cell yield × 10^{-6}/mouse				
	Total cells	Clara cells		Strong positive Clara cells	
		Number	Purity[b]	Number	Purity
1.040/1.089[c]					
Primary digest	3.62	1.41	39	0.65	18
	(± 1.32)	(± 0.51)	(± 3)	(± 0.25)	(± 5)
Major band	1.49	0.77	52	0.48	32
	(± 0.53)	(± 0.28)	(± 7)	(± 0.23)	(± 9)
1.01/1.04/1.06[d]					
Primary digest	2.51	0.70	28	NR	NR
1.02 band	0	0	0	0	0
1.04 band	0.40	0.21	52	0.20	49
1.06 band	2.30	0.53	23	0.46	20

[a]Cells of the primary digest are derived using 0.25% trypsin (15 min) from the lungs of mice with body weight 25–32 g; NR = not recorded.

[c]Data from 98 mice and 15 separate isolations; range (± SD) values are shown.

[d]Data from a single isolation.

[b]Purity is expressed as a percent of Clara cells as a proportion of the total cells present in the fraction.

Table 6. Purification of Clara cells from a primary lung digest using continuous Percoll gradient.[a]

Sample/fraction	Cell yield × 10^{-6}/mouse				
	Total cells	Clara cells		Strongly positive Clara cells	
		Number	Purity	Number	Purity
Primary digest	4.94	1.88	38	1.53	31
Band above 1.016	0	0		0	
Band 1.016–1.047	0.63	0.33	53	0.28	44
Band 1.047–1.070	1.90	1.10	58	0.97	51
Band 1.070–1.100	0.63	0.14	23	0.06	10
Band 1.100–1.142	0.83	0		0	

[a]Gradient prepared by centrifuging 1.058 density Percoll at 25,000g for 30 min at 4°C on SE 55.2 T_i rotor (angle 23°) in Beckman L8M ultracentrifuge. Density gradients from 1.016 to > 1.142 were verified by the use of marker beads. Data is from a single isolation with pooled material from six mice. Five milliliters of solution B containing 50 μg/mL DNase and 29.64 × 10^6 cells (primary digest) was layered and centrifuged on the preformed gradient at 800g for 30 min at 4°C.

weakly staining with NBT. Thus, of the 0.65 × 10^6 strongly NBT positive (functionally competent) Clara cells/mouse present in the primary digest, 0.48 × 10^6 (74%) were recovered in the major band (Table 5). Purity was not improved when cells were collected from a discontinuous gradient layered with Percoll of three different densities (1.02/1.04/1.06) (Table 5).

Primary digest cells were also purified using a continuous gradient (Table 6). In the single experiment carried out, 1.57 × 10^6 Clara cells/mouse were recovered (83%) in the different Percoll bands from the 1.88 × 10^6 Clara cells/mouse present in the primary digest. The greatest number of Clara cells, 1.10 × 10^6/mouse (58% pure), were found in a cell population in the density band 1.047 to 1.070, and practically all of these cells stain intensely with NBT (Table 6). The data suggest that the recovery of Clara cells, the purity of the populations, and the intensity of staining with NBT were all enhanced with the

use of a continuous Percoll gradient, in comparison with a discontinuous gradient (Tables 5 and 6). However, in view of the limited study conducted with the continuous gradient, such a conclusion may be premature.

It is evident that either gradient system permitted a limited selective purification of the Clara cells. Electron microscopy of the primary digest showed the presence of a number of different cell types although Clara cells were prominent (Fig. 2). Following separation on a discontinuous Percoll gradient, the cells present in the major band (1.040/1.089 interface) were mostly Clara cells, often in groups of 5 to 8 (Fig. 3). One major contaminating cell type was the ciliated cell (Fig. 4), although a number of small mononuclear cells were often present. The isolated Clara cells were of identical morphology to Clara cells *in situ* (*13,14*). They had a basal, strongly indented nucleus, an extensive smooth endoplasmic reticulum that is closely associated with secretary granules, and mitochondrialike bodies that have a diffuse granular matrix and indistinct cristae. Indeed, there is often considerable difficulty in distinguishing between secretory granules and the mitochondrialike bodies. In some cells translucent clefts are prominent (Fig. 5) that are not considered a fixation artifact. Some mitochondrialike bodies have a dense spherical structure in the matrix (Fig. 6) (*13*). Occasionally, some freshly isolated cells appear to be shedding their apical caps (Fig. 7) (*14,23*).

Sequential Protease Digestions to Isolate Clara Cells

The lung tissue remaining after a primary digestion (15–20 min, instilled 0.25% trypsin) was digested a second time for 20 to 30 min using different protease solutions. Cell preparations from both the primary and secondary digests in each experiment were purified on a discontinuous gradient, and the cellular composition of the major band was recorded (Table 7). The numbers of strongly NBT staining cells were also recorded as were the numbers of Type II cells in the sample. Type II cells were identified by their strong alkaline phosphatase activity (*19,22*), and Type II-rich isolates from rat lungs (*19*) were used as control preparations for the histochemical staining (Fig. 8). That these isolates contain very few Clara cells is shown by the limited NBT staining (Fig. 9).

Between 0.43 to 0.84 $\times$ 10^6 Clara cells/mouse could be purified (43–61% of the total cells) following a Percoll gradient of the primary digest. A large proportion of these cells (56–80%) were intensely stained with NBT, and few Type II cells (usually $< 1.0\%$) contaminated the preparation (Table 7). The second digestion with a protease enzyme released a much greater number of cells from the remaining lung tissue than that observed with the primary digestion. With the possible exception of the collagenase/dispase treatment, many of the cells present in the secondary digest were not recovered in the major band following discontinuous Percoll gradient formation. Greater numbers of Clara cells were often present in the major band from the second digestion process, but the purity of the cell sample was always lower than that achieved with the primary digest. In addition, fewer cells exhibited a strong NBT staining reaction in purified isolates from any secondary digestion process, and often increasing numbers of Type II cells were found to be present. Both of these effects were enhanced when increasing concentrations of trypsin were used in the secondary digestion. Collagenase/dispase (1%) released a large number of Clara cells in the second digestion, but only 8% of the cells stained intensely with NBT (Table 7, Fig. 10). The population of cells obtained was also highly contaminated with Type II cells (Table 7, Fig. 11). It was concluded from this series of experiments that little improvement in Clara cell yields could be achieved by the use of a second digestion. While a second digestion produced more cells, the preparations were of lower purity, contamination with Type II cells was more common, and the prolonged treatment of the tissue with a second enzyme probably reduced diaphorase activity and, thus, impaired the functional capacity of any Clara cells isolated.

Purification of Clara Cells by Direct Centrifugation

All of the procedures previously described required the production of a large number of cytospin samples that were always prepared in an identical manner (0.25×10^6 cells/chamber in a fluid volume of 2.5 mL, centrifugation at 1400 rpm at low acceleration setting for a total time of 6 min). It was noted that in the majority of preparations, and particularly those from primary digests, the distribution of the Clara cells throughout the area covered by the cytospin preparation was uneven. Clumps of cells and intensely staining cells were always located on one side of the cytospin area with many individual cells (weak or negative staining with NBT) being present in increasing number, the greater the distance from this Clara-rich area. It appeared that the cells had different sedimentation properties following centrifugation in cytospin buffer, and such a feature could be usefully employed to improve the purity of highly functional Clara cell preparations. Thus, a preliminary experiment was designed using a primary digest containing 29% Clara cells that was purified by either a discontinuous Percoll gradient as previously described, sedimentation at 0 °C for 2 hr or centrifugation for different time periods in a balanced salt solution containing DNase (Table 8). The best purification procedure (55% Clara cells, 0.64×10^6/mouse) was achieved in the pellet derived from centrifugation of the primary digest at 10 °C at 32*g* for 6 min. There was also a total recovery of the intensely staining Clara cells (Table 8). When the supernatant fraction from this centrifugation process was stained with NBT, it was confirmed that very few intensely staining

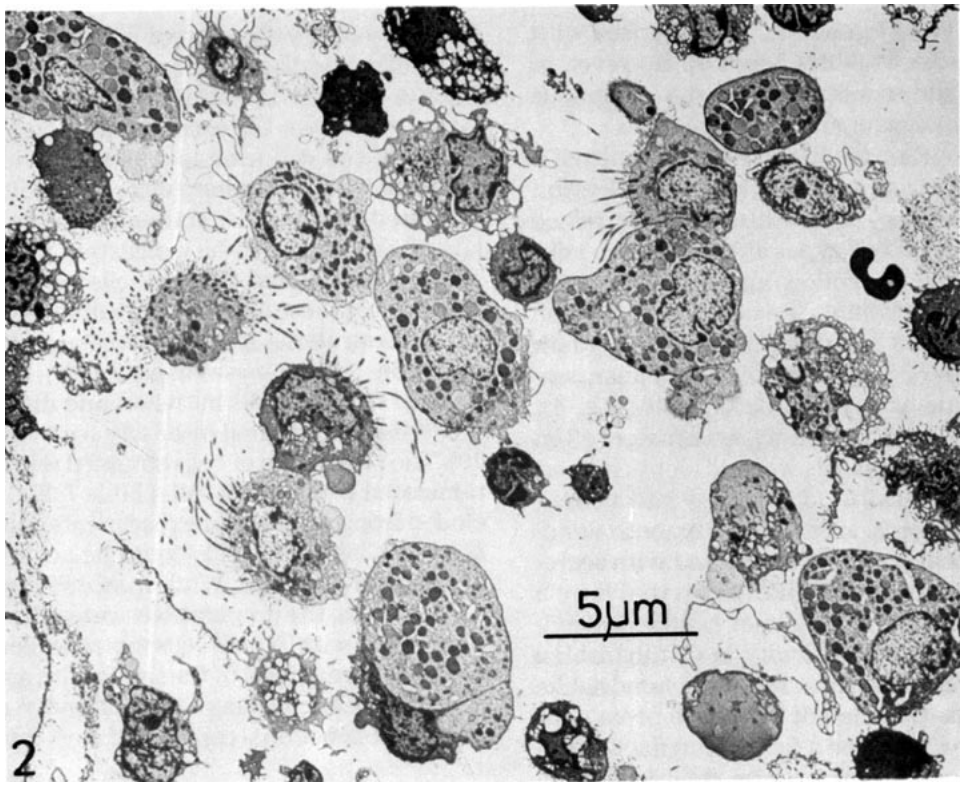

FIGURE 2. Electron micrograph of cells present in a primary digest of cells derived by trypsin instillation.

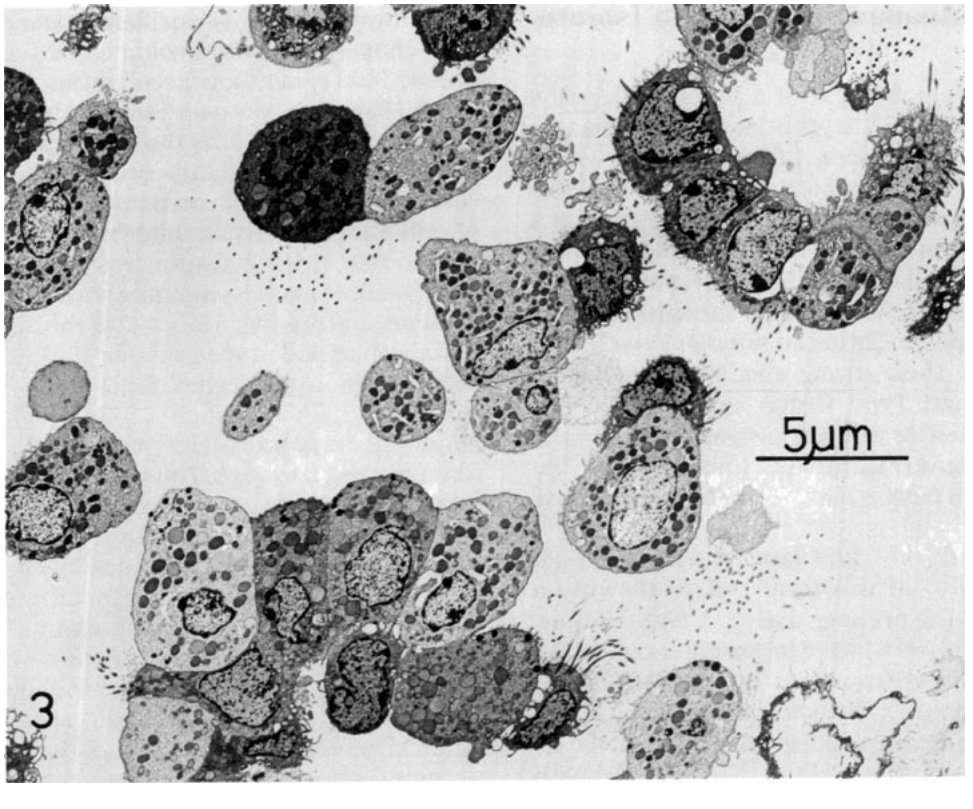

FIGURE 3. Electron micrograph of a cell population rich in Clara cells present in the major band of a discontinuous Percoll gradient.

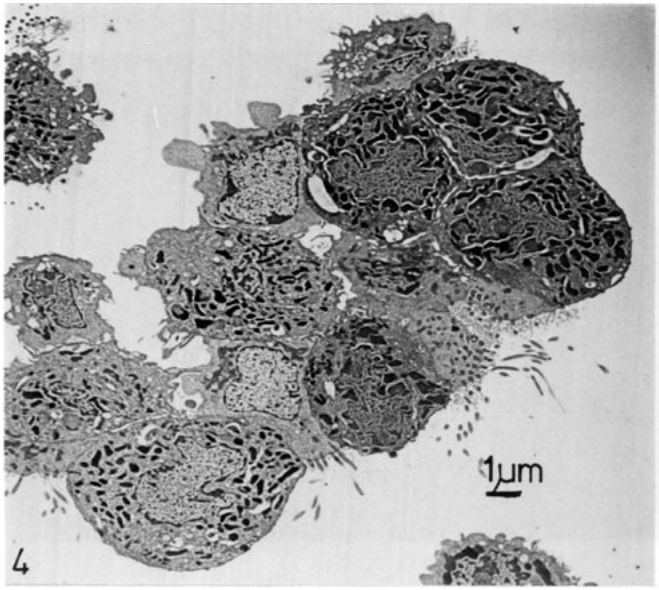

FIGURE 4. A mixture of ciliated and Clara cells present in groups in the major band of the discontinuous Percoll gradient.

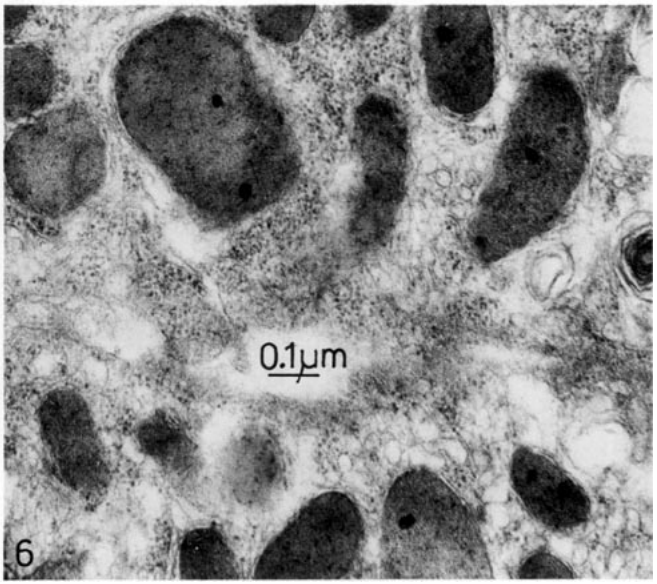

FIGURE 6. Mitochondriallike bodies of Clara cells, some of which contain a spherical structure within the granular matrix.

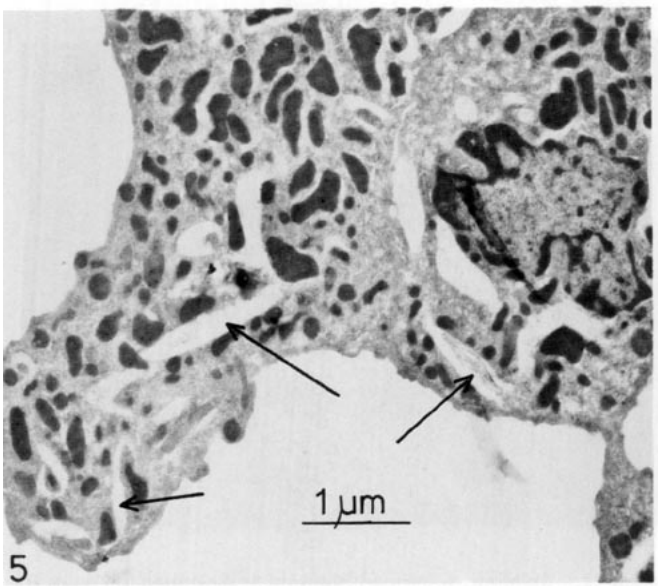

FIGURE 5. Prominent translucent clefts (arrows) are found in a number of isolated Clara cells.

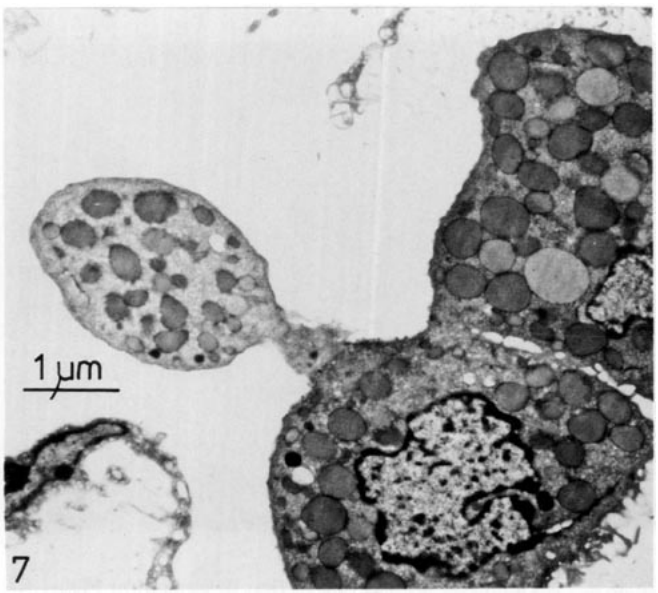

FIGURE 7. An isolated Clara cell that is shedding a portion of cytoplasm, probably derived from the apical region of the cell.

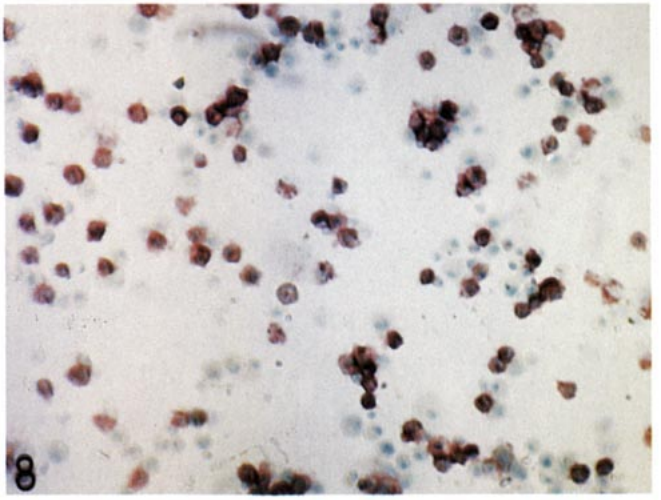

FIGURE 8. A cytospin preparation of an isolate rich in rat Type II cells (*19*) showing the strong alkaline phosphatase staining reaction (red). The preparation is counterstained with methylene green, ×160.

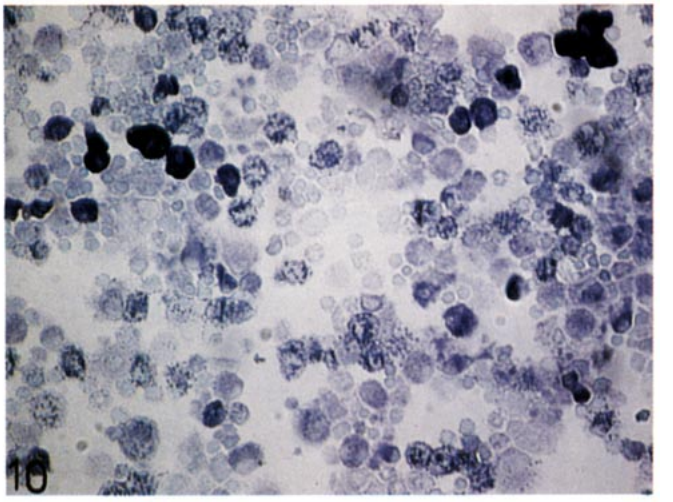

FIGURE 10. A cytospin preparation of cells of a secondary digest of mouse lung derived by collagenase/dispase (1%) and purified by Percoll gradient centrifugation. Weak staining with the NBT indicates that only a small number of functional Clara cells are present, ×250.

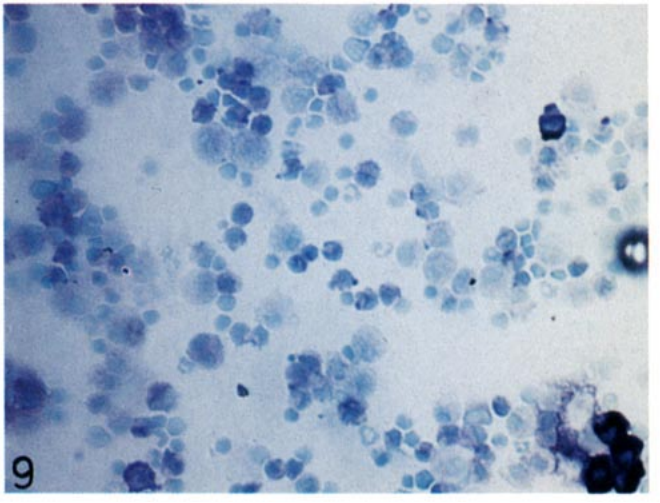

FIGURE 9. An identical preparation to that described for Figure 8 except that it is stained with NBT/methylene green. Only small numbers of Clara cells are present, ×250.

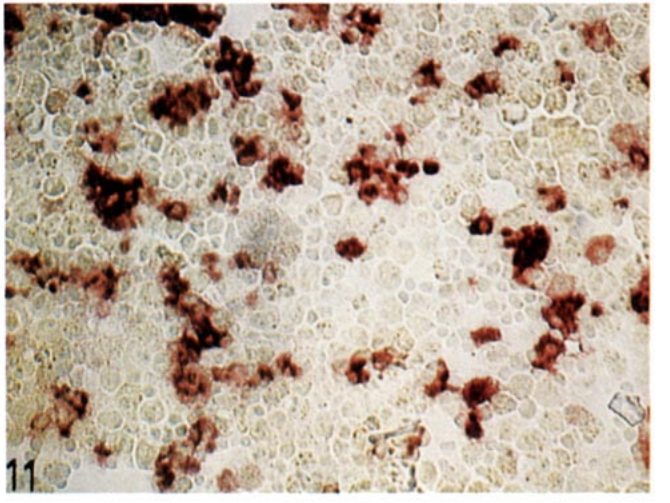

FIGURE 11. An identical preparation to that described in Figure 10 but stained for alkaline phosphatase. Numerous Type II cells are present in the sample, ×250.

Table 7. Release of Clara cells and Type II cells following primary digestion and secondary digestion of mouse lung tissue with instilled proteases followed by purification of cell suspensions on a Percoll gradient and collection of cells in the major band.

				Cell yields × 10^{-6}/mouse							
Protease used	Incubation time, min	n	PD or 2D	Total cells in digest	Total cells MB[a]	Clara cells, MB	Percent Clara cells, MB	Clara cells strong positive MB	Percent of strong positive, MB	Type II cells, MB	Percent Type II cells, MB
Trypsin, 0.25%	20	2	PD	4.10	1.37	0.84	61	0.67	49	0	0
Collagenase, 550 U/mL	20	2	2D	7.30	2.43	0.66	27	0.44	18	0.198	3.0
Trypsin, 0.25%	20	9	PD	3.88	1.47	0.63	43	0.40	27	0.063	1.0
Collagenase/dispase, 0.5%	20	6	2D	10.50	7.05	1.71	24	0.92	13	1.445	20.5
Collagenase/dispase, 1.0%	20	6	2D	9.01	7.25	1.22	16	0.62	8	1.349	25.5
Trypsin, 0.25%	15	9	PD	1.60	0.82	0.43	52	0.24	29	0	0
Elastase, 90 U/mL	20	3	2D	4.80	3.20	0.45	14	0.32	10	0.160	5.0
Dispase, 9 U/mL	20	3	2D	4.10	3.20	0.83	26	0.51	16	0.160	5.0
Trypsin, 0.25%	15	24	PD	3.11	1.16	0.57	50	0.30	26	0.015	1.3
Trypsin, 0.50%	30	3	2D	4.53	2.57	1.03	40	NR	NR	0.128	5.0
Trypsin, 0.75%	30	5	2D	6.68	3.30	0.65	20	0.27	8	0.231	7.0
Trypsin, 1.00%	30	5	2D	7.02	2.85	0.76	26	0.10	4	0.200	7.0
Trypsin, 1.50%	30	4	2D	10.10	3.30	0.44	13	0.19	6	0.495	15.0
Trypsin, 2.00%	30	2	2D	8.63	4.75	0.29	6	0.05	1	1.188	25.0

[a]MB = 1.040/1.089 interface; NR = not recorded

Table 8. A comparison of Clara cell purification from the primary digest (0.25% trypsin) by means of a discontinuous Percoll gradient, sedimentation at 0°C, or washing/centrifugation.[a]

		Clara cells		Strongly positive Clara cells	
Fraction/sample	Total cells × 10^{-6}/mouse	No. × 10^{-6}/mouse	Purity	No. × 10^{-6}/mouse	Purity
Primary digest	2.94	0.85	29	0.51	18
Major band 1.040/1.089 interface	2.05	0.78	38	0.58	28
Sedimentation (0°C)					
Pellet	1.18	0.50	42	0.41	35
Supernatant	1.74	0.33	19	0.16	9
Wash/centrifugation	1.5 min,				
Pellet	0. 88	0.33	37	0.25	28
Supernatant	2.06	0.45	22	0.29	14
Wash/centrifugation	3.0 min,				
Pellet	1.41	0.55	42	0.41	32
Supernatant	1.52	0.35	23	0.19	15
Wash/centrifugation	6.0 min,				
Pellet	1.18	0.64	55	0.52	44
Supernatant	1.76	0.09	5	0.02	1

[a]Cells from the same primary digest were purified by sedimentation on ice (2 hr), by layering on a Percoll gradient or by washing with solution B (containing 50 μg/mL DNase) and then light centifugation (32g, 10°C) for 1.5- to 6.0-min intervals.

Clara cells are lost in this fraction that contains a large number of unstained mononuclear cells. Reduction of the centrifugation time below 6 min resulted in an increase in the number of Clara cells present in the supernatant fraction. Sedimentation on ice for 2 hr gave a pellet of Clara cells that was equally pure (42%) to that derived by a Percoll gradient separation, although fewer cells were present in the sedimented sample.

The final method chosen to purify Clara cells from a primary trypsin digest of lung involved three washing/centrifugation steps (Table 9). The three centrifugation steps removed 54, 20, and 7%, respectively, of the total cells present in the primary digest (4.85 × 10^6/mouse). Very few of the cells in these supernatant fractions stained with NBT (Fig. 12), but a Clara-rich population of cells (68% NBT positive) were found in the pellet after the third centrifugation (Table 9, Fig. 13). Calculations show that 4.75 × 10^6 total cells can be accounted for from a starting population of 4.85 × 10^6 (98% recovery). However, the recovery of Clara cells exhibiting diaphorase activity in pellet three (0.55 × 10^6/mouse) from a starting population of 1.50 × 10^6, was relatively poor at 37%.

Table 9. Distribution of Clara cells following washing and light centrifugation of the primary protease digest.[a]

Fraction/sample	Cell yield $\times 10^{-6}$/mouse
Primary digest	
Total cells	4.85 (± 1.82)
Clara cells	1.50 (± 0.60)
% Clara	31 (± 5)
Supernatant 1	
Total cells	2.63 (± 0.76)
Clara cells	0.17 (± 0.06)
% Clara	6 (±) 1)
Supernatant 2	
Total cells	0.95 (± 0.43)
Clara cells	0.06 (± 0.02)
% Clara	6 (± 0)
Supernatant 3	
Total cells	0.36 (± 0.04)
Clara cells	0.01 (± 0)
% Clara	3 (± 0)
Pellet 3	
Total cells	0.81 (± 0.20)
Clara cells	0.55 (± 0.16)
% Clara	68 (± 8)

[a]Full details are given in text; mean values (± SD) are shown from three separate isolations, $n = 28$.

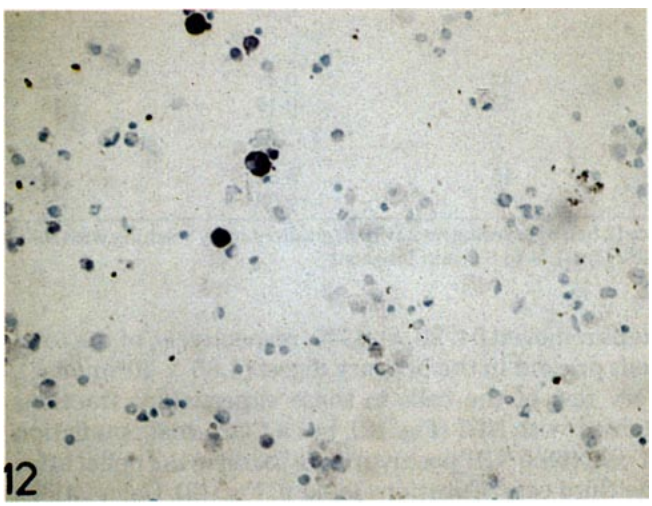

FIGURE 12. Cells present in supernatant 1, derived by centrifugation of the primary trypsin digest at 32*g* for 6 min. Few of the cells stain strongly positive with NBT indicating that few functional Clara cells are removed in this fraction, ×160.

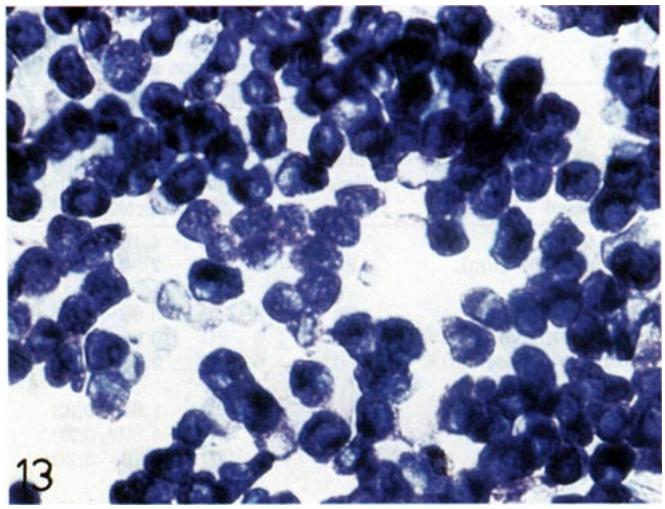

FIGURE 13. NBT staining of the cells in pellet 3, derived by washing and centrifuging the primary trypsin digest (see text). This sample represents a highly purified preparation of Clara cells, ×400.

Thus, a number of Clara cells lost diaphorase activity during the washing centrifugation procedure, an effect noticed previously with the Percoll separations. Monooxygenase (P-450) activity was monitored in a number of Clara cell preparations and found to be comparable to that reported previously for rabbit Clara cells (*8*). The purified Clara cell pellet from the mouse had approximately twice the 7-ethoxycoumarin deethylase and an identical coumarin hydroxylase activity to that reported for rabbit Clara cells (Table 10) (*8*). As might be predicted, P-450 activity was lower in the primary digest (containing 21% Clara cells) and absent in the supernatant population derived from the first centrifugation that only has 4% Clara cells (Table 10).

Clara cells purified by the washing/centrifugation (Figure 14) may be used directly for analysis or toxicity studies or be plated in culture. In some instances a second purification procedure may be undertaken by placing

Table 10. Monooxygenase activity in Clara cell preparations.

Cell sample	Percent of Clara cells in sample	7-Ethyoxy-coumarin deethylase[a]	Coumarin hydroxylase[a]
Primary digest	21	60 (10.3)	8.4 (1.5)
Pellet 3[b]	65	251 (59.5)	42.0 (9.8)
Supernatant 1[c]	4	ND[d]	ND

[a]Enzyme activities are expressed as picomole hydroxycoumarin produced/mg protein/min (figures in parentheses are moles produced/10^6 cells/min). Enzyme activities were measured by a modification of the method of Challiner et al. (*24*) whereby 0.5–1.5 × 10^6 cells were incubated in 0.5 mM (ethoxycoumarin or coumarin) substrate in 66 mM Tris-HCl containing 0.2 mM NADPH.

[b]Pellet 3 is the purified Clara cell fraction following three centrifugations/washings (see text).

[c]Supernatant 1 is the cell population which remains in suspension after centrifugation of the primary digest at 32*g* for 6 min.

[d]ND = not detected.

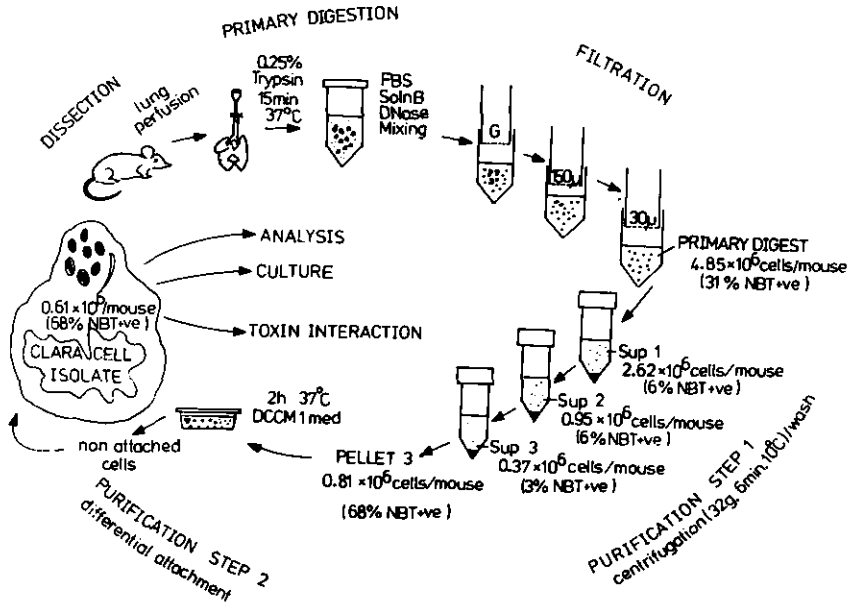

FIGURE 14. A summary diagram outlining a recommended method for the isolation of mouse Clara cells.

the cell populations (derived from pellet 3) in DCCM 1 culture medium for 2 hr at 37 °C in a gas phase of 95% air/5% CO_2. Some contaminating cells (NBT negative) do attach to the plastic substrata, while Clara cells (strongly NBT positive) do not adhere during this time. However, despite the fact that as many as 25% of the cells in the population adhered to the substrata in 2 hr the proportion of NBT positive cells in the nonadherent Clara cell-rich population did not increase (68%). Thus, it appears that over 2 hr in culture, Clara cells may lose diaphorase activity.

Conclusions

A method has been described for the isolation of functional Clara cells of high purity (70%) from the mouse by a simple technique of washing/centrifugation of a primary trypsin digest. All the methods previously published require the use of an elutriator and produce Clara isolates of 45 to 70% purity. Most previous studies have concentrated on isolating rabbit Clara cells, of which approximately 1 to 2 × 10^6 are obtained per animal. This relatively poor yield of cells from a lung of approximately 10 g wet wt may be attributed to a number of factors, including the relative impurity of the primary digest from rabbit (Clara cells account for only 5% of this population). The subsequent elutriation procedure, however, also involved a considerable loss of Clara cells (see "Introduction"), possibly because only individual cells are likely to be separated by this method. Thus, Clara cells isolated in clumps or those that form groups by reaggregating in suspension may be lost upon elutriation. Extensive disaggregation of clumps of Clara cells (and ciliated cells) by prolonged enzymatic digestion is likely to lead to an enhancement in the loss of functional (diaphorase) activity. One particularly important requirement in isolating Clara cells for culture or toxicity studies is that they should retain a high P-450 activity.

The method described above for the mouse indicates that approximately 0.5 × 10^6 functionally competent Clara cells with P-450 activity can be obtained from each individual animal. Furthermore, the population of cells is not contaminated with epithelial Type II cells that also contain monooxygenase activity. The fact that mouse Clara cells are located mainly in the bronchioles and are easily removed by trypsin probably explains why the yield of cells per gram wet weight of lung far exceeds that achieved with the rabbit. It is anticipated that a number of improvements of the method described for the mouse should be forthcoming, and preventing the loss of diaphorase activity during the isolation procedure would represent a useful advance.

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